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DATE: Wednesday, April 18, 2007

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<input type="checkbox"/>	L4	L3 same (complex or groels or groesl or gro-esl or pspabcde or dnakj or hsparpoh)	116
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<input type="checkbox"/>	L6	colaco.in.	116
<input type="checkbox"/>	L7	L6 and l1	9
<input type="checkbox"/>	L8	L6 and 049704	0
<input type="checkbox"/>	L9	L6 and vaccine	37
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L11: Entry 53 of 56

File: USPT

Sep 28, 1999

DOCUMENT-IDENTIFIER: US 5958416 A

TITLE: Heat shock protein peptides and methods for modulating autoimmune central nervous system disease

Detailed Description Text (17):

While not meant to limit the invention, it is believed that the mycobacterial heat shock proteins stimulate immune responses that immunologically cross-react with self antigenic components of myelin such as CNP. These cross-reactive immune responses contribute to the damage and development of central nervous system autoimmune disease. Immunization with a peptide derived from CNP can protect against development of allergic encephalomyelitis stimulated by a complex mixture of encephalitogenic antigens in a spinal cord preparation. It is believed that peptides derived from mycobacterial or bacterial heat shock proteins that immunologically cross-react with myelin proteins such as CNP are involved in stimulating an immune response that results in a final common pathway to disease development. Cross-reactive or homologous peptides derived from either CNP or HSP65 can be useful to stimulate an immune response that blocks or inhibits a pathogenic immune response to central nervous system components.

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20050232946. 10 Jun 03. 20 Oct 05. Vaccine against microbial pathogens. Colaco, Camilo Anthony Leo Selwyn. 424/199.1; 424/200.1 A61K039/12 A61K039/02.

☐ 2. 20050175635. 23 Aug 02. 11 Aug 05. Method and composition. Colaco, Camilo Anthony Leo Selwyn. 424/234.1; A61K039/38.

☐ 3. 20030113900. 07 Aug 02. 19 Jun 03. Methods of preserving prokaryotic cells and compositions obtained thereby. Tunnacliffe, Alan G., et al. 435/252.1; C12N001/20.

☐ 4. 6468782. 04 Dec 97; 22 Oct 02. Methods of preserving prokaryotic cells and compositions obtained thereby. Tunnacliffe; Alan G., et al. 435/260; 435/244. C12N001/04.

☐ 5. WO 200163278A. Identifying candidate vaccine antigenic fragments by extracting stress-induced proteins from pathogenic organisms treated with stress-inducing stimuli, identifying antigenic fragments associated with stress proteins. COLACO, C A L S. A61K039/00 A61K039/38 A61P031/00 A61P033/00 C07K014/195 C07K014/44 G01N033/50.

☐ 6. WO 200114411A. Producing an immunogenic complex for use in pestivirus subunit vaccines, having a heat shock protein coupled to a heterologous polypeptide antigen, comprises expressing the antigen in a cell subjected to heat shock stimulus. COLACO, C A L S, et al. A61K039/12 A61K039/15 A61K039/385 A61K039/44 A61K047/42 A61P031/00 A61P031/12 A61P037/02 C07K000/00 C07K002/00 C07K014/005 C07K014/435 C07K014/48 C07K019/00 C12N015/09 C12N015/12 C12N015/866 C12P021/02 C12P021/02 C12R001/93.

☐ 7. WO 200113944A. Production of a vaccine against extracellular parasites, useful e.g. against bacteria or fungi, comprises using stress-induced products from the parasite as antigenic determinants. COLACO, C A L S. A61K000/00 A61K039/00 A61K039/002 A61K039/005 A61K039/02 A61K039/04 A61K039/09 A61K039/108 A61K039/395 A61P031/00 A61P031/04 A61P031/10 A61P033/02 C07K014/315 C07K016/12 C12N001/21 C12N015/31 C12Q001/68 G01N033/569.

☐ 8. WO 200113943A. Production of vaccine against intracellular parasites, useful e.g. against mycobacteria or malaria, using endogenous stress-induced products as antigenic determinant. COLACO, C A L S, et al. A61K000/00 A61K039/00 A61K039/002 A61K039/02 A61K039/385 A61P031/04 A61P033/02.

☐ 9. WO 200010597A. Production of a vaccine comprises treating virally infected cells, recombinant or cancerous cells with cytokine to produce heat shock-related stress proteins, which can then be used in the vaccines. COLACO, C A L S, et al. A61K000/00 A61K038/00 A61K038/21 A61K039/00 A61K039/12 A61P037/02 C07K001/00 C07K014/435 C07K014/47.

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- ☐ 1. [20050276846](#). 20 May 05. 15 Dec 05. Solid dose delivery vehicle and methods of making same. Roser, Bruce J., et al. 424/464; 514/2 A61K038/17 A61K009/20.
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- ☐ 2. [20050276845](#). 20 May 05. 15 Dec 05. Solid dose delivery vehicle and methods of making same. Roser, Bruce J., et al. 424/464; 514/3 A61K038/28 A61K009/20.
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- ☐ 3. [20050276759](#). 20 May 05. 15 Dec 05. Solid dose delivery vehicle and methods of making same. Roser, Bruce J., et al. 424/46; 514/2 A61K038/17 A61K009/14 A61L009/04.
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- ☐ 4. [20050163750](#). 18 Mar 05. 28 Jul 05. Methods of preventing aggregation of various substances upon rehydration or thawing and compositions obtained thereby. Roser, Bruce J., et al. 424/85.2; 424/85.4 514/171 514/2 514/3 514/44 514/53 A61K038/20 A61K038/22 A61K038/21 A61K031/7012 A61K048/00 A61K038/28 A61K031/56.
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- ☐ 5. [20040219206](#). 28 May 04. 04 Nov 04. Solid dose delivery vehicle and methods of making same. Roser, Bruce J., et al. 424/464; 514/23 514/53 514/61 A61K031/70 A61K031/715 A61K009/20.
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- ☐ 6. [20040052825](#). 29 Aug 03. 18 Mar 04. Solid dose delivery vehicle and methods of making same. Roser, Bruce J., et al. 424/400; 514/171 514/53 514/54 514/61 A61K031/715 A61K031/56 A61K031/573.
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- ☐ 7. [20030147961](#). 27 Feb 03. 07 Aug 03. Solid dose delivery vehicle and methods of making same. Roser, Bruce J., et al. 424/486; 424/204.1 424/234.1 A61K039/12 A61K039/02 A61K009/14.
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- ☐ 8. [20030054040](#). 25 Oct 02. 20 Mar 03. Solid dose delivery vehicle and methods of making same. Roser, Bruce J., et al. 424/488; A61K009/14.
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- ☐ 9. [20020012687](#). 31 Aug 01. 31 Jan 02. Solid dose delivery vehicle and methods of making same. Roser, Bruce J., et al. 424/423; A61F002/00.
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- ☐ 10. [20020009464](#). 06 Aug 01. 24 Jan 02. Modified glycosides, compositions comprised thereof and methods of use thereof. Colaco, Camilo. 424/204.1; 359/885 424/94.1 536/123 A61K038/43 C07H001/00 C07H003/00 A61K039/12 G02B005/22.
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- ☐ 11. [20010055583](#). 16 Apr 01. 27 Dec 01. Methods of preventing aggregation of various substances upon rehydration or thawing and compositions obtained thereby. Roser, Bruce J., et al. 424/85.2; 424/130.1 424/85.4 514/171 514/2 514/4 514/44 514/53 A61K038/28 A61K038/20 A61K038/21 A61K038/22 A61K031/7016.
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- ☐ 12. [20010038858](#). 05 Jan 01. 08 Nov 01. Solid delivery systems for controlled release of molecules incorporated therein and methods of making same. Roser, Bruce J., et al. 424/488; 514/23 514/53 A61K009/14 A61K031/7016 A61K031/70.
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- ☐ 13. [7056495](#). 29 Aug 03; 06 Jun 06. Solid dose delivery vehicle and methods of making same. Roser; Bruce J., et al. 424/45; 424/423 424/427 424/430 424/434 424/436 424/449 424/451 424/46 424/464 424/489 424/78.04. A61L9/04 20060101.

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- ☐ 14. 6893657. 27 Feb 03; 17 May 05. Solid dose delivery vehicle and methods of making same. Roser; Bruce J., et al. 424/451; 424/423 424/427 424/45 424/46 424/464 424/489 424/78.04 430/434 430/436 430/449. A61K009/48 A61K009/20 A61K009/14 A61F002/02 A61F006/06 .
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- ☐ 15. 6890512. 16 Apr 01; 10 May 05. Methods of preventing aggregation of various substances upon rehydration or thawing and compositions obtained thereby. Roser; Bruce J., et al. 424/1.29; 424/1.11 424/1.33 424/489 424/499. A61K051/00 .
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- ☐ 16. 6811792. 25 Oct 02; 02 Nov 04. Solid dose delivery vehicle and methods of making same. Roser; Bruce J., et al. 424/423; 424/443 424/449 424/502. A61F002/02 A61K013/02 A61K009/70 A61K009/50 .
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- ☐ 17. 6586006. 05 Jan 01; 01 Jul 03. Solid delivery systems for controlled release of molecules incorporated therein and methods of making same. Roser; Bruce J., et al. 424/484; 424/423 424/426 424/443 424/449 424/502 514/777 514/781. A61K009/14 A61K009/70 A61K009/50 A61K047/00 A61F002/00 .
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- ☐ 18. 6565871. 31 Aug 01; 20 May 03. Solid dose delivery vehicle and methods of making same. Roser; Bruce J., et al. 424/423; 424/427 424/430 424/434 424/436 424/449 424/45 424/46 424/78.04. A61F002/02 A61F006/06 A61F013/02 A61L009/04 A61K031/74 .
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- ☐ 19. 6517860. 30 Dec 97; 11 Feb 03. Methods and compositions for improved bioavailability of bioactive agents for mucosal delivery. Roser; Bruce J., et al. 424/434; 424/45 424/450 514/53 514/958 536/119. A61F013/00 .
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- ☐ 20. 6331310. 01 Aug 00; 18 Dec 01. Solid dose delivery vehicle and methods of making same. Roser; Bruce J., et al. 424/423; 424/443 424/449 424/502. A61F002/02 A61K013/02 A61K009/70 A61K009/50 .
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- ☐ 21. 6290991. 02 Dec 94; 18 Sep 01. Solid dose delivery vehicle and methods of making same. Roser; Bruce J., et al. 424/502; 424/500 424/501. A61K009/50 .
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- ☐ 22. 5621094. 08 Jun 94; 15 Apr 97. Method of preserving agarose gel structure during dehydration by adding a non-reducing glycoside of a straight-chain sugar alcohol. Roser; Bruce J., et al. 536/114; 435/6 536/120 536/4.1. C12Q001/68 C07G003/00 C07G017/00 C07H015/04 .
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- ☐ 23. EP001212349A1. 18 Aug 00. 12 Jun 02. RECOMBINANT SUBUNIT VACCINE. SHANNON, ANTHONY DOUGLAS, et al. C07K002/00; C07K019/00 A61K039/12 A61K039/15 A61K039/44 A61K047/42 A61P037/02 C12N015/12 C12N015/866.
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- ☐ 24. WO 200220045A. Novel vaccine for eliciting an immune response in an animal, comprises a microbial pathogen which has been subjected to stress inducing stimuli, as an immunogenic determinant. COLACO, C A L S, et al. A61K000/00 A61K039/002 A61K039/02 A61K039/04 A61K039/106 A61K039/112 A61K039/12 A61P031/04 A61P037/04.
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- ☐ 25. WO 200113942A. Producing vaccine from prokaryotic cells having increased trehalose content, useful for protection against bacterial infections, and has increased immunogenicity and stability. COLACO, C A L S. A61K039/00 A61K039/002 A61K039/02 A61P031/04 A61P031/10 A61P033/02.
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☐ 26. US20020009464A. New modified glycoside(s) having good solvent properties - useful, e.g. in solid delivery systems for dissolution, encapsulation, storage and delivering therapeutic molecules. COLACO, C. A61K009/00 A61K009/02 A61K009/14 A61K009/20 A61K009/50 A61K009/70 A61K031/7032 A61K031/7105 A61K031/711 A61K038/00 A61K038/43 A61K039/00 A61K039/12 A61K039/39 A61K045/00 A61K047/26 C07H000/00 C07H001/00 C07H003/00 C07H015/04 G02B005/22.

☐ 27. WO 9824882A. Preservation of prokaryotic cells - by increasing intracellular trehalose concentration, mixing with a stabilising agent, and drying to produce a glass form of the stabilising agent. COLACO, C, et al. A61K035/66 A61K035/74 A61K039/02 C12N000/00 C12N001/04 C12N001/20 C12N001/21 C12N015/09.

☐ 28. EP 541556B. Stabilisation using sugars of their derivs. - of biological substances or organic cpds. e.g. proteins. COLACO, C, et al. A61K047/26 A61K047/36 C07B063/04 C07G003/00 C07G017/00 C07H015/04 C07K001/02 C07K003/00 C12N009/96 C12Q001/68.

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L11: Entry 9 of 56

File: PGPB

Mar 2, 2006

DOCUMENT-IDENTIFIER: US 20060045888 A1

TITLE: Antigen library immunization

Description of Disclosure:

[0093] The bacterial antigens that can be improved by DNA shuffling for use as vaccines also include, but are not limited to, *Helicobacter pylori* antigens CagA and VacA (Blaser (1996) Aliment. Pharmacol. Ther. 1: 73-7; Blaser and Crabtree (1996) Am. J. Clin. Pathol. 106: 565-7; Censini et al. (1996) Proc. Nat'l. Acad. Sci. USA 93: 14648-14643). Other suitable *H. pylori* antigens include, for example, four immunoreactive proteins of 45-65 kDa as reported by Chatha et al. (1997) Indian J. Med. Res. 105: 170-175 and the *H. pylori* GroES homologue (HspA) (Kansau et al. (1996) Mol. Microbiol. 22: 1013-1023). Other suitable bacterial antigens include, but are not limited to, the 43-kDa and the fimbriin (41 kDa) proteins of *P. gingivalis* (Bouts et al. (1996) Oral Microbiol. Immunol. 11: 236-241); pneumococcal surface protein A (Briles et al. (1996) Ann. NY Acad. Sci. 797: 118-126); *Chlamydia psittaci* antigens, 80-90 kDa protein and 110 kDa protein (Buendia et al. (1997) FEMS Microbiol. Lett. 150: 113-9); the chlamydial exoglycolipid antigen (GLXA) (Whittum-Hudson et al. (1996) Nature Med. 2: 1116-1121); *Chlamydia pneumoniae* species-specific antigens in the molecular weight ranges 92-98, 51-55, 43-46 and 31.5-33 kDa and genus-specific antigens in the ranges 12, 26 and 65-70 kDa (Halme et al. (1997) Scand. J. Immunol. 45: 378-84); *Neisseria gonorrhoeae* (GC) or *Escherichia coli* phase-variable opacity (Opa) proteins (Chen and Gotschlich (1996) Proc. Nat'l. Acad. Sci. USA 93: 14851-14856), any of the twelve immunodominant proteins of *Schistosoma mansoni* (ranging in molecular weight from 14 to 208 kDa) as described by Cutts and Wilson (1997) Parasitology 114: 245-55; the 17-kDa protein antigen of *Brucella abortus* (De Mot et al. (1996) Curr. Microbiol. 33: 26-30); a gene homolog of the 17-kDa protein antigen of the Gram-negative pathogen *Brucella abortus* identified in the nocardioform actinomycete *Rhodococcus* sp. NI86/21 (De Mot et al. (1996) Curr. Microbiol. 33: 26-30); the staphylococcal enterotoxins (SEs) (Wood et al. (1997) FEMS Immunol. Med. Microbiol. 17: 1-10), a 42-kDa *M. hyopneumoniae* NrdF ribonucleotide reductase R2 protein or 15-kDa subunit protein of *M. hyopneumoniae* (Fagan et al. (1997) Infect. Immun. 65: 2502-2507), the meningococcal antigen PorA protein (Feavers et al. (1997) Clin. Diagn. Lab. Immunol. 3: 444-50); pneumococcal surface protein A (PspA) (McDaniel et al. (1997) Gene Ther. 4: 375-377); *F. tularensis* outer membrane protein FopA (Fulop et al. (1996) FEMS Immunol. Med. Microbiol. 13: 245-247); the major outer membrane protein within strains of the genus *Actinobacillus* (Hartmann et al. (1996) Zentralbl. Bakteriol. 284: 255-262); p60 or listeriolysin (Hly) antigen of *Listeria monocytogenes* (Hess et al. (1996) Proc. Nat'l. Acad. Sci. USA 93: 1458-1463); flagellar (G) antigens observed on *Salmonella enteritidis* and *S. pullorum* (Holt and Chaubal (1997) J. Clin. Microbiol. 35: 1016-1020); *Bacillus anthracis* protective antigen (PA) (Ivins et al. (1995) Vaccine 13: 1779-1784); *Echinococcus granulosus* antigen 5 (Jones et al. (1996) Parasitology 113: 213-222); the *rol* genes of *Shigella dysenteriae* 1 and *Escherichia coli* K-12 (Klee et al. (1997) J. Bacteriol. 179: 2421-2425); cell surface proteins Rib and alpha of group B streptococcus (Larsson et al. (1996) Infect. Immun. 64: 3518-3523); the 37 kDa secreted polypeptide encoded on the 70 kb virulence plasmid of pathogenic *Yersinia* spp. (Leary et al. (1995) Contrib. Microbiol. Immunol. 13: 216-217 and Roggenkamp et al. (1997) Infect. Immun. 65: 446-51); the OspA (outer surface protein A) of the Lyme disease spirochete *Borrelia burgdorferi* (Li et al. (1997) Proc. Nat'l. Acad. Sci.

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L11: Entry 10 of 56

File: PGPB

Feb 16, 2006

DOCUMENT-IDENTIFIER: US 20060036301 A1

TITLE: System method and apparatus for localized heating of tissue

Brief Summary Text:

[0047] The host immune system can be activated against infectious disease by heat shock protein chaperoned peptides in a manner similar to the effect seen against metastatic tumors. Heat shock proteins chaperoning peptides derived from both viral and bacterial pathogens have been shown to be effective at creating immunity against the infectious agent. For infectious agents for which efficacious vaccines are not currently available (especially for intracellular pathogens e.g. viruses, Mycobacterium tuberculosis or Plasmodium) HSP chaperoned peptides may be useful for the development of novel vaccines. It is expected that purified HSP chaperoned peptides (e.g. gp96 complexes) used as vaccines for diseases caused by highly polymorphic infectious agents would be less effective against genetically distinct pathogen populations. For a summary of past work on HSP vaccines against infectious agents, see generally: [0048] (23) Neiland, Thomas J. F., M. C. Agnes A. Tan, Monique Monnee-van Muijen, Frits Koning, Ada M. Kruisbeek, and Grada M. van Bleek, "Isolation of an immunodominant viral peptide that is endogenously bound to stress protein gp96/GRP94." Proc. Nat'l Acad. Sci. USA, 93: 6135-6139 (1996). [0049] (24) Heikema, A., Agsteribbe, E., Wilschut, J., Huckriede, A., "Generation of heat shock protein-based vaccines by intracellular loading of gp96 with antigenic peptides." Immunology Letters, 57: 69-74. (1997) [0050] (25) Zugel, U., Sponaas, A. M., Neckermann, J., Schoel, B., and Kaufmann, S. H. E., "gp96-Peptide Vaccination of Mice Against Intracellular Bacteria." Infection and Immunity, 69: 4164-4167 (2001). [0051] (26) Zugel, U., and Kaufmann, S. H. E., "Role of Heat Shock Proteins in Protection from and Pathogenesis of Infectious Diseases." Clinical Microbiology Reviews, 12: 19-39 (1999).

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L5: Entry 17 of 57

File: PGPB

Oct 6, 2005

DOCUMENT-IDENTIFIER: US 20050221395 A1

TITLE: Methods and products based on oligomerization of stress proteins

Summary of Invention Paragraph:

[0002] In modern medicine, immunotherapy or vaccination has virtually eradicated diseases such as polio, tetanus, tuberculosis, chicken pox, measles, hepatitis, etc. The approach using vaccinations has exploited the ability of the immune system to prevent infectious diseases. Vaccination with non-live materials such as proteins generally leads to an antibody response or CD4+ helper T-cell response. Raychaudhuri and Morrow (1993) Immunology Today 14:344-348. On the other hand, vaccination or infection with live materials such as live cells or infectious viruses generally leads to a CD8+ cytotoxic T-lymphocyte (CTL) response. A CTL response is crucial for protection against cancers, infectious viruses and certain bacteria. This poses a practical problem, for the only way to achieve a CTL response is to use live agents, which are themselves pathogenic. The problem is generally circumvented by using attenuated viral and bacterial strains or by killing whole cells which can be used for vaccination. These strategies have worked well but the use of attenuated strains always carries the risk that the attenuated agent may recombine genetically with host DNA and turn into a virulent strain. Thus, there is need for methods that can lead to CD8+ CTL response by vaccination with non-live materials such as proteins in a specific manner. It has been discovered that heat shock protein-peptide complexes have particular utility as vaccines against cancers and infectious diseases. (Srivastava et al., (1994) Curr. Op. Immu. 6:728; Srivastava (1993) Adv. Cancer Res. 62:153).

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Adonis

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L11: Entry 31 of 56

File: PGPB

May 1, 2003

DOCUMENT-IDENTIFIER: US 20030082232 A1

TITLE: CALCIUM PHOSPHATE DELIVERY VEHICLE AND ADJUVANT

Summary of Invention Paragraph:

[0004] Traditional vaccines have typically used complex immunogens such as inactivated viruses or bacteria to evoke immunity. Such vaccines were often associated with adverse side effects (e.g. granuloma formation, pyrogenicity, and hypersensitivity). The use of subunit vaccines has reduced the number and severity of unwanted side effects associated with vaccines produced with more complex immunogens. Subunit vaccines are comprised of only one, or a few, proteins or polysaccharides from the target pathogen. Suzue et al in Experientia (77, 1996, pp451-465) teach heat shock proteins (HSPs) as subunit vaccines. Due to their small size, by themselves, subunit vaccines tend to be only weakly immunogenic, often failing to induce a satisfactory level of immunity. Thus to be effective, the use of subunit vaccines require strategies to enhance immunogenicity, such as the use of enhanced adjuvants and specific delivery strategies.

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Immune responses to stress proteins: Applications to infectious disease and cancer





Author: Mizzen L.¹

Source: Biotherapy, Volume 10, Number 3, 1998, pp. 173-189(17)

Publisher: Springer

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Abstract:

Heat shock proteins, or stress proteins have been identified as part of a highly conserved cellular defence mechanism mediated by multiple, distinct gene families and corresponding gene products. As intracellular chaperones, stress proteins participate in many essential biochemical pathways of protein maturation and function active during times of stress and during normal cellular homeostasis. In addition to their well-characterized role as protein chaperones, stress proteins are now realized to possess another important biological property: immunogenicity. Stress proteins are now understood to play a fundamental role in immune surveillance of infection and malignancy and this body of basic research has provided a framework for their clinical application. As key targets of both humoral and cellular immunity during infection, stress proteins have accordingly received considerable research interest as prophylactic vaccines for infectious disease applications. The unique and potent immunostimulatory properties of stress proteins have similarly been applied to the development of new approaches to cancer therapy, including both

09/613,303

- determinants and its involvement in autoimmunity and cancer. *Clin. Immunol. Immunopathol.* 73:283-289.
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 198. Multhoff, G., and L. E. Hightower. 1996. Cell surface expression of heat shock proteins and the immune response. *Cell Stress Chaperones* 1:167-176.
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ABSTRACT:

The present invention relates to a method for isolating and identifying specific immunogenic pathogen-specific peptides associated with stress proteins induced by

the treatment of pathogens and pathogen-infected cells with stress inducing stimuli. The invention also relates to the use of the antigenic fragments derived from complexes thereof with heat shock or other stresses proteins as the immunogenic determinant in vaccine compositions.

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20050232946. 10 Jun 03. 20 Oct 05. Vaccine against microbial pathogens. Colaco, Camilo Anthony Leo Selwyn. 424/199.1; 424/200.1 A61K039/12 A61K039/02.

☐ 2. 20050175635. 23 Aug 02. 11 Aug 05. Method and composition. Colaco, Camilo Anthony Leo Selwyn. 424/234.1; A61K039/38.

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☐ 4. 6468782. 04 Dec 97; 22 Oct 02. Methods of preserving prokaryotic cells and compositions obtained thereby. Tunnacliffe; Alan G., et al. 435/260; 435/244. C12N001/04 .

☐ 5. WO 200163278A. Identifying candidate vaccine antigenic fragments by extracting stress-induced proteins from pathogenic organisms treated with stress-inducing stimuli, identifying antigenic fragments associated with stress proteins. COLACO, C A L S. A61K039/00 A61K039/38 A61P031/00 A61P033/00 C07K014/195 C07K014/44 G01N033/50.

☐ 6. WO 200114411A. Producing an immunogenic complex for use in pestivirus subunit vaccines, having a heat shock protein coupled to a heterologous polypeptide antigen, comprises expressing the antigen in a cell subjected to heat shock stimulus. COLACO, C A L S, et al. A61K039/12 A61K039/15 A61K039/385 A61K039/44 A61K047/42 A61P031/00 A61P031/12 A61P037/02 C07K000/00 C07K002/00 C07K014/005 C07K014/435 C07K014/48 C07K019/00 C12N015/09 C12N015/12 C12N015/866 C12P021/02 C12P021/02 C12R001:93.

☐ 7. WO 200113944A. Production of a vaccine against extracellular parasites, useful e.g. against bacteria or fungi, comprises using stress-induced products from the parasite as antigenic determinants. COLACO, C A L S. A61K000/00 A61K039/00 A61K039/002 A61K039/005 A61K039/02 A61K039/04 A61K039/09 A61K039/108 A61K039/395 A61P031/00 A61P031/04 A61P031/10 A61P033/02 C07K014/315 C07K016/12 C12N001/21 C12N015/31 C12Q001/68 G01N033/569.

☐ 8. WO 200113943A. Production of vaccine against intracellular parasites, useful e.g. against mycobacteria or malaria, using endogenous stress-induced products as antigenic determinant. COLACO, C A L S, et al. A61K000/00 A61K039/00 A61K039/002 A61K039/02 A61K039/385 A61P031/04 A61P033/02.

☐ 9. WO 200010597A. Production of a vaccine comprises treating virally infected cells, recombinant or cancerous cells with cytokine to produce heat shock-related stress proteins, which can then be used in the vaccines. COLACO, C A L S, et al. A61K000/00 A61K038/00 A61K038/21 A61K039/00 A61K039/12 A61P037/02 C07K001/00 C07K014/435 C07K014/47.

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Heat shock protein-peptide complexes in cancer immunotherapy.

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Heat shock proteins (HSPs) are associated with a broad spectrum of peptides derived from the cells from which they are isolated. Vaccination with such HSP-peptide complexes elicits protective immunity against tumors or other cells used as the source of HSPs. These observations suggest that HSP-peptide complexes are suitable as vaccines against cancers and infectious diseases.

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ABSTRACT:

The present invention provides a vaccine comprising a microbial pathogen, wherein the microbial pathogen is subjected to a stress inducing stimuli. The stress inducing stimuli can be heat or osmotic stress, through preferably the microbial pathogen is genetically modified such that at least one repressor gene for a heat shock protein gene is inactivated, thus allowing the constitutive expression of heat shock proteins. In particular the use of heat shock protein repressor mutant bacteria is shown to be effective for inducing immunity when comprised within vaccines of the present invention. The present invention further provides a method for producing a vaccine comprising stressed induced microbial pathogens and further the use of the heat shock protein repressor deletion mutant microbes as vaccine vectors which can be additionally allow the expression of heterologous antigen fragments.

Immunity against *Yersinia enterocolitica* by Vaccination with *Yersinia* HSP60 Immunostimulating Complexes or *Yersinia* HSP60 plus Interleukin-12

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Microbial heat shock proteins (HSP) are dominant antigens for the host immune response. Because of the high sequence homology between mammalian and microbial HSP, their value as component of a subunit vaccine has been the subject of controversy. Previous work from this laboratory, however, demonstrated for the first time that the adoptive transfer of HSP60-reactive CD4⁺ $\alpha\beta$ T-cell clones confers protection against bacterial infection in mice but does not induce autoimmunity. In the present study, we have therefore evaluated the potential role of *Yersinia* HSP60 (Y-HSP60) as a vaccine in the *Yersinia enterocolitica* mouse infection model. For this purpose, immunostimulating complexes (ISCOM) which included Y-HSP60 were constructed. Parenteral administration of this vaccine induced high Y-HSP60-specific serum antibody responses as well as T-cell responses. This reaction was paralleled by immunity against a lethal challenge with *Y. enterocolitica*. In contrast, mucosal application of Y-HSP60-ISCOM failed to induce systemic Y-HSP60-specific T-cell responses and thus failed to induce immunity against yersiniae. Likewise, vaccination with purified recombinant Y-HSP60 induced antibody responses but only weak T-cell responses. Therefore, this vaccination protocol was not protective. However, when interleukin-12 was used as an adjuvant, purified Y-HSP60 induced significant Y-HSP60-specific T-cell responses and thus induced protection against subsequent challenge with yersiniae. These studies suggest that (i) microbial HSP might be promising candidates for the design of subunit vaccines and (ii) interleukin-12 is an efficient alternative adjuvant to ISCOM particles for induction of protective CD4 Th1-cell-dependent immune responses against bacterial pathogens.

Infection with *Yersinia enterocolitica* causes a broad spectrum of clinical manifestations including enteritis, enterocolitis, and mesenteric lymphadenitis (12, 14). Moreover, both systemic infections with abscesses and granuloma-like lesions in the liver and spleen and immunopathological sequelae such as reactive arthritis and erythema nodosum are associated with *Y. enterocolitica* infections (1, 58, 62). The pathogenicity of *Y. enterocolitica* for humans and rodents depends on plasmid-encoded (e.g., Yops, YadA) and chromosomally encoded (e.g., *Yersinia* bactin, Inv) virulence factors (13). Several of these virulence factors mediate efficient resistance against phagocytosis and complement lysis and thus promote the extracellular survival of *Y. enterocolitica* in infected host tissues (13, 26, 37). In fact, electron-microscopic studies confirmed that *Y. enterocolitica* is extracellularly located in Peyer's patch and liver tissue of experimentally infected mice (4, 24, 37).

An overwhelming body of evidence suggests that a specific T-cell-mediated host response is required for resolution of *Y. enterocolitica* infections (3, 7, 8). Both CD4 Th1 and CD8 T cells confer resistance against this pathogen when transferred into T-cell-deficient athymic nude mice or naive syngeneic mice (3, 7, 8). As the cytokines interleukin-12 (IL-12), tumor necrosis factor alpha, and gamma interferon are essential mediators of the protective events against yersiniae, macrophages are possibly the final (T-cell-activated) effector cells in the infection process (2, 5, 10, 11). The integrins Mac-1 and VLA-4 are involved not only in phagocytosis of *Yersinia* cells by mac-

rophages in vitro and in vivo but also in host cell interactions; thus, they play a multifunctional role in host defense mechanisms against *Y. enterocolitica* (6).

The 60-kDa *Yersinia* heat shock protein (Y-HSP60) is a major antigen for murine T and B cells (49). Protection of mice against *Y. enterocolitica* infection can be transferred by Y-HSP60-reactive T cells but not by Y-HSP60-reactive antibodies (49). Moreover, immunodominant 12- and 13-amino-acid T-cell epitopes of Y-HSP60, which are presented by MHC class II (I-A) molecules, have been recently identified (48). Whether Y-HSP60 might be useful as a vaccine, however, remained questionable, since microbial HSP were believed to be involved in autoimmune responses (23, 27, 29, 32, 33, 63). Indeed, there is extensive sequence homology between mammalian and microbial HSP (23, 27, 29, 33, 38, 63). Hence, an immune response against shared epitopes of microbial and mammalian HSP might destroy the tolerance for self-antigens (HSP) and subsequently might cause autoimmune diseases (23, 27, 29, 31, 33, 63). In keeping with this hypothesis, T cells isolated from the synovial fluid of patients with *Yersinia*-triggered reactive arthritis recognize mycobacterial HSP65 (27). Further, the cell surface of gamma interferon-stressed macrophages contains endogenous HSP exposed to autologous cytotoxic T cells, which subsequently can lyse the stressed macrophages (36).

The primary aim of the present study was to evaluate whether Y-HSP60 might be a useful vaccine. Since T cells have been identified as major protective component of the host response against *Y. enterocolitica*, a Y-HSP60 vaccine should efficiently stimulate T cells. Immunostimulating complexes (ISCOM) have been used as immunization vectors for the induction of a wide range of immune responses to a variety of protein antigens (41, 43, 45). ISCOM contain Quil A, an ex-

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tract from the bark of the South American tree *Quillaja saponaria* Molina with adjuvant properties (34). Further elements of ISCOM are cholesterol, phosphatidylcholine, and the desired antigen in equimolar amounts (39). In this study, we produced and used Y-HSP60-ISCOM as a vaccine. Alternatively, pure Y-HSP60 with recombinant IL-12 as an adjuvant was used for vaccination in a second approach, since cytokines turned out to be useful as adjuvants for vaccines (57, 59). The results reported herein indicate that both vaccination strategies conferred protection against *Y. enterocolitica* infection and argue for a possible role of HSP as a component of an efficient (subunit) vaccine.

MATERIALS AND METHODS

Bacteria and infection of animals. *Y. enterocolitica* WA-314 serotype O8 was cultured as described previously (7). Female C57BL/6 and BALB/c mice, 6 to 8 weeks old, were purchased from Charles River Wiga, Sulzfeld, Germany, and kept in positive-pressure cabinets under specific-pathogen-free conditions. The mice were intravenously infected with 5 to 10 50% lethal doses (LD₅₀) of *Y. enterocolitica* WA-314 as described previously (7). At various intervals after infection, mice were killed, their spleens were removed, and serum was collected. The number of bacteria present in the spleens of infected mice was determined by homogenization of the spleens in phosphate-buffered saline (PBS; pH 7.4) containing 0.5% bovine serum albumin (Biomol, Hamburg, Germany) and 0.5% Tergitol TMN10 (Fluka, Buchs, Switzerland) and plating of 0.1 ml of serial dilutions of the homogenate on Mueller-Hinton agar. CFU were counted after 2 days of incubation at 27°C. All experiments were repeated at least three times for verification.

Antigen preparations. Y-HSP60 was cloned and purified as described elsewhere (49). Briefly, *Escherichia coli* M15(pAN5) (49) was grown, and expression of recombinant Y-HSP60 was induced with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, Deisenhofen, Germany). Y-HSP60 was extracted and purified by affinity chromatography on metal-chelating Ni resin (Pharmacia LKB, Uppsala, Sweden) in the presence of 8 M urea. After renaturation, the protein was sterilized, its concentration was measured by the bicinchoninic acid assay (Pierce, Oud-Beijerland, The Netherlands), and aliquots were frozen at -20°C until further use. The purity of Y-HSP60 preparation was controlled by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting (49). Heat-killed *Yersinia* were prepared as described elsewhere (7). Ovalbumin (OVA; Sigma) was used as the control antigen.

Preparation of ISCOM by incorporation of acid-treated proteins. Lyophilized Y-HSP60 or ovalbumin (OVA) (20 mg) was dissolved in PBS at a final concentration of 2 mg of protein per ml. To each 1 ml of protein solution was added 100 μl of 1 M glycine buffer (pH 2.5), which exposed hydrophobic amino acid residues by reducing the pH of the protein solution. The 20 μl of a lipid mix consisting of 8.3 mg of cholesterol (Sigma) per ml, 8.3 mg of phosphatidylcholine (Sigma) per ml, 167 mg of decanoyl-N-methylglucamide (Mega10; Sigma) per ml, and 0.17% (vol/vol) chloroform was added. Afterwards, Quil A (Roth, Karlsruhe, Germany) was added at a final concentration of 1 mg/ml. Then the mixture was sonicated for 15 min in a 20°C water bath to disrupt any protein aggregates. After incubation for 60 min at 20°C, the solution was extensively dialyzed against 0.1 M glycine buffer (pH 2.5) for 20 h at 20°C and then against PBS for 20 h at 20°C and for at least 20 h at 4°C. Formation of the typical cage-like structures of ISCOM was determined by transmission electron microscopy. For this purpose, Y-HSP60-ISCOM and OVA-ISCOM were negatively stained with 0.5% uranyl acetate (Merck) at 4°C for 16 h and analyzed by electron microscopy. Typical ISCOM particles form cage-like structures 30 to 40 nm in diameter. Furthermore, incorporation of antigen into ISCOM was analyzed by density sucrose gradient centrifugation as described elsewhere (47).

Vaccination protocols. (i) **Immunization with ISCOM.** To induce proliferative T-cell responses in mice (four animals per group), immunizations with either Y-HSP60-ISCOM or OVA-ISCOM were performed by using three different protocols. (j) On days 0, 10, and 21, the mice were given intraperitoneal (i.p.) injections of 10 μg of Y-HSP60-ISCOM or OVA-ISCOM in 0.1 ml of PBS. (ii) On days 0, 10, and 21 the mice were given injections in the footpads with 10 μg of Y-HSP60-ISCOM or OVA-ISCOM in 0.05 ml of PBS. (iii) On days 0, 7, 14, and 21, 100 μg of Y-HSP60-ISCOM or OVA-ISCOM was orally administered 0.1 ml of PBS via a 0.86-mm-diameter polypropylene tube connected to a syringe fitted with a 20-gauge 1/2 needle.

Eight days after the final injection, the mice were killed, sera were collected, and splenic or lymph nodes T cells were prepared to characterize humoral and cellular immune responses induced by the immunization procedures.

For protection assays, mice (four animals per group) were given i.p. injections on days 0, 14, and 28 with 10 μg of Y-HSP60-ISCOM or OVA-ISCOM in 0.1 ml of PBS or with 0.1 ml of PBS. Ten days later (day 38), the mice were infected intravenously (i.v.) with a lethal dose (5 to 10 LD₅₀) of *Y. enterocolitica*. Seven days after the infection, the mice were killed and sera were collected. Spleens were removed and homogenized, and serial dilutions of the homogenates were

plated on Mueller-Hinton agar as described above. Bacterial numbers in the spleens of mice are given as log₁₀ CFU.

(iii) **Immunization with Y-HSP60 plus IL-12.** To induce proliferative T-cell responses, mice were given injections of 0.2 μg of recombinant murine IL-12 (r-IL-12; kindly provided by M. Gately, Hoffmann-La Roche Inc., Nutley, N.J.) and 50 μg of purified Y-HSP60 i.p. on days 0 and 3. Control mice received Y-HSP60 alone (50 μg), r-IL-12 alone (0.2 μg), or 0.1 ml of PBS. Five days later, splenic T cells were isolated, purified, and assayed for their reactivity with different antigens.

For protection assays, mice (three or four animals per group) were given i.p. or subcutaneous (s.c.) injections in the footpads with (i) 25 μg of Y-HSP60 plus 0.5 μg of murine r-IL-12, (ii) 25 μg of Y-HSP60, (iii) 0.5 μg of r-IL-12, or (iv) PBS on days 0 and 14. Fourteen days later, the mice were infected i.v. with a lethal dose of *Y. enterocolitica* (~10 LD₅₀). Six days after infection, the mice were killed, sera were collected, spleens were homogenized, and serial dilutions were plated on Mueller-Hinton agar to determine bacterial counts in the spleens.

Flow cytometry. To characterize the purity of isolated T cells, the cells were stained with fluorescein isothiocyanate-coupled anti-CD3ε (145 2C11) monoclonal antibody. Staining procedures were carried out as previously described (49). Finally, fluorescence staining of cells was analyzed with a FACScan (Becton Dickinson, Heidelberg, Germany). Dead cells were excluded from analysis by gating out propidium iodide (Sigma)-positive cells. At least 10,000 cells per sample were analyzed.

T-cell preparation and proliferative responses. At 5 to 8 days after immunization of mice, spleens and/or popliteal lymph nodes were removed and single-cell suspensions were prepared as described previously (7). T cells were isolated and purified by Ficoll density gradient centrifugation and passed through a nylon wool column (30). The purity of eluted T cells was controlled by flow cytometry as described above. The final cell fraction usually contained 90% CD3⁺ T cells.

For proliferation assays, 1×10^5 to 2×10^5 freshly prepared and purified T cells were incubated with 2×10^5 irradiated (3,000 rads) syngeneic splenic cells as antigen-presenting cells and 1 to 10 μg of antigen per ml of culture medium in a total of 0.2 ml in 96-well microtiter plates (Nunc, Wiesbaden, Germany). The culture medium was Click-RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 2 mM L-glutamine, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 5×10^{-5} M 2-mercaptoethanol, 10 μg of streptomycin per ml, 100 U of penicillin per ml, and 10% heat-inactivated fetal calf serum (Biochrom). Heat-killed whole bacterial cells of *Y. enterocolitica* O8 (7), recombinant purified Y-HSP60, truncated recombinant Y-HSP60 (amino acid residues 90 to 286) (49), and cyanogen bromide-cleaved Y-HSP60 peptides (48) were used at 10 μg/ml of culture medium. Synthetic Y-HSP60 peptides (amino acid residues 194 to 232 and 74 to 86) (48) were used at 1 μg/ml. Concanavalin A (3 μg/ml) was used as an indication of maximal T-cell stimulation, and background activity was determined in the absence of antigen (medium, antigen-presenting cells, and T cells).

After 3 days of incubation, microcultures were pulsed with [³H]thymidine. Eight hours later, samples were collected with a cell harvester (Pharmacia) and [³H]thymidine uptake was determined with a liquid scintillation counter (Pharmacia) (7). Proliferative responses were expressed as stimulation index (SI), which was calculated as follows: $SI = \frac{[^3H]thymidine\ uptake\ (cpm)\ in\ the\ presence\ of\ the\ indicated\ antigen}{[^3H]thymidine\ uptake\ (cpm)\ without\ antigen}$. Standard deviations are not included in the tables for the sake of clarity. All experiments were repeated at least three times for verification.

Detection of serum antibodies by ELISA. Sera of immunized and control mice were prepared and analyzed for the presence of Y-HSP60 or OVA-specific immunoglobulin G (IgG) antibodies as described previously (61). Recombinant Y-HSP60 or OVA (50 μg; 10 μg/ml of PBS) was used as the antigen for coating. Sera were diluted 1:100 in PBS containing 0.5% Tween 20 (Merck, Darmstadt, Germany). Alkaline phosphatase-conjugated anti-mouse IgG (Sigma) was diluted 1:1,000 with PBS containing 0.5% Tween 20. p-Nitrophenylphosphate disodium (Sigma) was used as the substrate. Optical density was measured with an enzyme-linked immunosorbent assay (ELISA) reader (Flow Laboratories, Meckenheim, Germany) at a wavelength of 405 nm. Five duplicates of sera from uninfected nonimmunized control mice were tested as negative controls to obtain cutoff values. The cutoff value was defined as the mean value for the negative controls plus 2 standard deviations. As a positive control, a Y-HSP60-specific monoclonal antibody was used (49).

Statistics. Data from different experimental groups were compared by Student's *t* test. $P < 0.05$ were considered statistically significant.

RESULTS

Vaccination of mice with Y-HSP60-ISCOM particles. C57BL/6 mice were immunized i.p. or s.c. three times with 10 μg of Y-HSP60-ISCOM (Fig. 1) or OVA-ISCOM. Alternatively, mice were immunized by four oral administrations of 100 μg of Y-HSP60-ISCOM or OVA-ISCOM. Eight days after administration of the final boost, the mice were killed, serum and splenic or lymph node T cells were prepared and

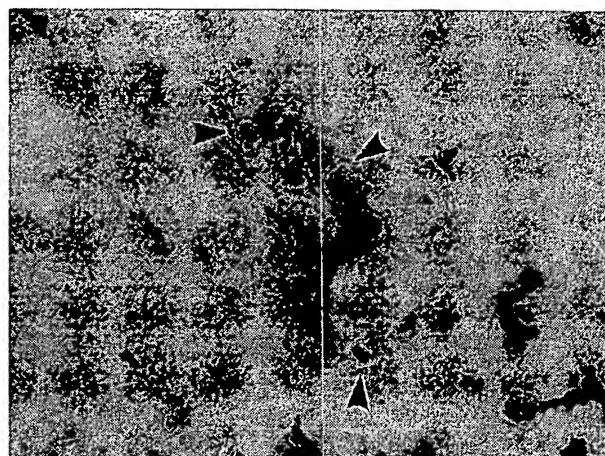


FIG. 1. Transmission electron micrographs of ISCOM containing Y-HSP60. The particles have formed the typical cage-like structure (arrows). Magnification, $\times 40,000$.

serum antibody responses and T-cell responses against Y-HSP60 or OVA were determined. Both i.p. (Fig. 2) and s.c. (data not shown) immunization induced a strong antigen-specific serum IgG antibody response against Y-HSP60 or OVA, respectively (Fig. 2). In contrast, orally or nasally immunized mice did not show an antigen-specific response (data not shown).

Furthermore, proliferative T-cell responses upon stimulation with various *Yersinia* antigens or OVA were determined. T cells from mice immunized with Y-HSP60-ISCAM exhibited a significant proliferative response upon stimulation with either heat-killed yersiniae or various Y-HSP60 preparations (Table 1). Likewise, Y-HSP60 peptides (amino acid residues 74 to 86 and 194 to 232) which have been identified as dominant T-cell epitopes for *Yersinia*-reactive CD4 Th1 cells from C57BL/6 mice were recognized by T cells after i.p. immunization with Y-HSP60-ISCAM. In contrast, T cells isolated from mice after orogastric immunization did not show significant reactivity upon antigenic stimulation. Furthermore, T cells isolated from

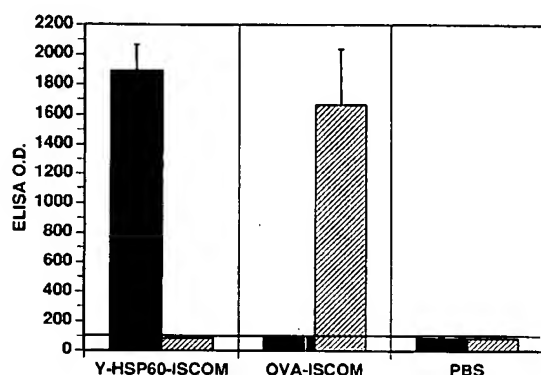


FIG. 2. Serum IgG antibody responses of immunized C57BL/6 mice 10 days after the final i.p. injection with $10 \mu\text{g}$ of Y-HSP60-ISCAM or OVA-ISCAM or $100 \mu\text{l}$ of PBS buffer on days 0, 14, and 28. The ELISA was performed, and the coating antigens (Y-HSP60 [solid bars] or OVA [hatched bars]) were prepared and used as described in Materials and Methods. Sera were diluted 1:100. Bars represent means and standard deviations of results obtained with four animals. The horizontal line indicates the cutoff value. O.D., optical density.

TABLE 1. Proliferative T-cell responses after immunization with ISCOM particles^a

Antigen ^b	SI ^c			OVA- ISCOM (i.p.)
	Y-HSP60-ISCOM			
	i.p.	s.c.	p.o. ^d	
HKY	4.8	5.0	1.8	1.9
Y-HSP60	5.0	1.9	<1	1.1
Y-HSP60 (aa 90-286)	6.2	2.3	1.6	1.9
Y-HSP60/CNBr	7.3	2.9	1.7	1.5
Y-HSP60 peptides				
aa 194-232	5.0	1.7	1.0	1.3
aa 74-86	4.0	1.8	1.1	1.6
OVA	1.0	1.1	<1	2.1

^a Preparation of ISCOM and immunization of mice were performed as described in Materials and Methods.

^b T cells were incubated with $10 \mu\text{g}$ of HKY; full-length, truncated (aa 90 to 286), or CNBr-cleaved Y-HSP60 or OVA per ml of medium; or $1 \mu\text{g}$ of the depicted synthetic peptides per ml of medium.

^c Proliferative responses were determined by [^3H]thymidine uptake of 2×10^5 T cells after antigenic stimulation in the presence of 2×10^5 irradiated syngeneic antigen-presenting cells. Proliferative responses are expressed as the SI.

^d p.o., oral administration.

mice immunized with OVA-ISCAM responded upon stimulation with OVA only but not with Y-HSP60 (Table 1).

To determine whether the above immunization procedure induced protection against *Yersinia* infection, mice that had been immunized i.p. with Y-HSP60-ISCAM or OVA-ISCAM were challenged i.v. with 10 LD_{50} of *Y. enterocolitica*, and 7 days later, the bacterial counts in their spleens were determined. The data depicted in Fig. 3 indicate that immunization with Y-HSP60-ISCAM induced significant protection against subsequent challenge with yersiniae whereas administration of OVA-ISCAM did not. Hence, bacterial counts in the spleens of control mice (administration of PBS or OVA-ISCAM) were $\sim 1,000$ -fold higher ($P < 0.05$). Taken together, these results demonstrate that Y-HSP60-ISCAM immunization (footpad or i.p.) induced Y-HSP60-specific T- and B-cell responses, the former of which conferred subsequent protection against a lethal challenge with yersiniae.

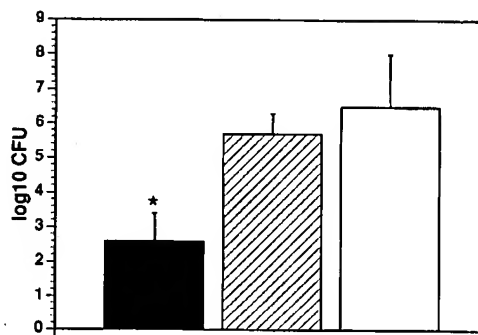


FIG. 3. Bacterial numbers in spleens of immunized C57BL/6 mice after infection with *Y. enterocolitica*. For immunization, mice were given i.p. injections on days 0, 14 and 28 with $10 \mu\text{g}$ of ISCOM preparations (Y-HSP60-ISCAM [solid bars] or OVA-ISCAM [hatched bars]) or with PBS (open bars) as a control. The mice were infected i.v. with 2.2×10^4 CFU of *Y. enterocolitica* 10 days later. Seven days after infection, the mice were killed, spleens were removed and homogenized, and bacterial counts were determined. Bacterial numbers in the spleens are expressed as \log_{10} CFU. Bars represent means and standard deviations of results obtained with four animals. The asterisk indicates statistically significant differences between experimental and control groups.

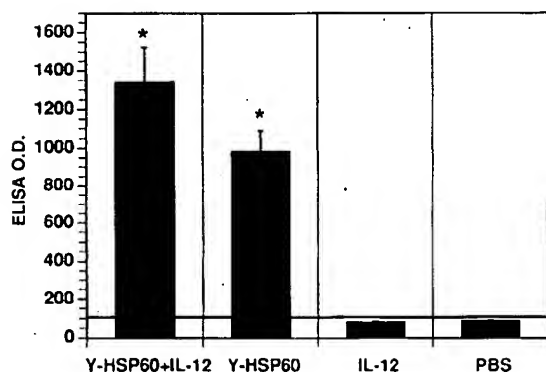


FIG. 4. Serum IgG antibody responses of BALB/c mice 14 days after the final s.c. immunization with Y-HSP60 plus r-IL-12, Y-HSP60, r-IL-12, or PBS on days 0 and 14. The ELISA was performed, and the coating antigen (Y-HSP60) was prepared and used as described in Materials and Methods. Sera were diluted 1:100. Bars represent means and standard deviations of results obtained with three animals. The horizontal line indicates the cutoff value. O.D., optical density. The asterisk indicates statistically significant differences between experimental and control groups.

Vaccination with Y-HSP60 plus IL-12 as adjuvant. Recent studies suggested that ILs might be useful as adjuvant for immunization (57, 59). Since IL-12 turned out to be a crucial cytokine for the development of *Yersinia*-specific T-cell responses in mice, pure recombinant Y-HSP60 was administered with IL-12 as adjuvant. For this purpose, mice were given two i.p. or s.c. injections of (i) 0.2 to 0.5 μ g of r-IL-12 plus 25 to 50 μ g of Y-HSP60, (ii) 25 to 50 μ g of Y-HSP60 without r-IL-12, or (iii) r-IL-12 only. As described above, serum antibody responses and T-cell responses were determined 5 days after the boost injection. Immunization with Y-HSP60 plus IL-12 or with Y-HSP60 only induced significant Y-HSP60-specific serum IgG antibody responses, whereas injection of IL-12 alone or PBS buffer did not (Fig. 4). However, Y-HSP60-specific IgG antibody responses were significantly higher in mice which received the combination of Y-HSP60 plus IL-12 than in those which received Y-HSP60 only ($P < 0.05$).

Analysis of proliferative T-cell responses revealed that immunization with Y-HSP60 plus IL-12 induced significant proliferative responses upon stimulation with heat-killed yersiniae or various Y-HSP60 preparations (Table 2). However, there was only a weak response upon stimulation with Y-HSP60 peptides (data not shown). Likewise, T cells from mice immu-

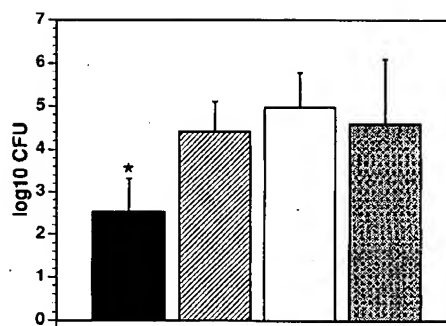


FIG. 5. Bacterial numbers in spleens of immunized BALB/c mice after infection with *Y. enterocolitica*. For immunization, the mice were given injections into the footpads on days 0 and 14 with 25 μ g of Y-HSP60 plus 0.5 μ g of IL-12 (solid bar), 25 μ g of Y-HSP60 (hatched bar), 0.5 μ g of IL-12 (open bar), or PBS (stippled bar). The mice were infected i.v. with 4.1×10^3 CFU of *Y. enterocolitica* 14 days later. Six days after infection, the mice were killed, spleens were homogenized, and bacterial counts were determined. Bacterial numbers in the spleens were expressed as log₁₀ CFU. Bars represent means and standard deviations of results obtained with three or four animals. The asterisk indicates statistically significant differences between experimental and control groups.

nized with Y-HSP60 without IL-12 as adjuvant exhibited Y-HSP60-specific proliferative responses, although the responses were weaker than those induced by the combination of Y-HSP60 with IL-12.

Subsequently, mice were challenged i.v. 14 days after two footpad immunizations with viable *Y. enterocolitica* to determine whether this immunization protocol induced protection. The data depicted in Fig. 5 indicate that only administration of Y-HSP60 plus IL-12 induced significant protection whereas immunization with Y-HSP60 or injection with IL-12 alone did not confer protection. Hence, bacterial counts in spleens were reduced ~100-fold in the former group only ($P < 0.05$). Comparable results were obtained after i.p. immunization (data not shown). These results show that IL-12 can be used as an alternative adjuvant to induce Y-HSP60-specific protective immune responses against *Y. enterocolitica* in mice.

DISCUSSION

In the past decade, efforts have been made toward the development of subunit vaccines. The rational basis of such vaccines is to include only proteins or peptides which efficiently elicit protective immune responses and exclude components of the antigen that account for deleterious immune responses. The limited antigenic composition of a subunit vaccine, however, might be inefficient in individuals with certain major histocompatibility complex MHC haplotypes according to the MHC restriction of peptide presentation for T cells (52).

Since microbial HSP have been recognized as immunodominant antigens for the host response, a controversial discussion about their significance as vaccines arose for two major reasons. First, HSP-specific immune responses may induce autoimmune diseases by generating immune responses against shared epitopes in the conserved HSP sequences and hence cross-reactions between foreign (microbial) and self (endogenous) HSP (23, 27, 29, 32, 33, 63). Second, on the other hand, there is evidence that because of their chaperone properties, HSP themselves may have immunostimulating properties (9). For instance, HSP may act as carrier molecules for other antigens (55), accelerate the formation of compact class II molecules (15), and channel exogenous antigens into the endogenous pathway (55). Furthermore, there is some evidence that

TABLE 2. Proliferative T-cell responses of after immunization with Y-HSP60 plus IL-12 as an adjuvant^a

Antigen ^b	SI ^c for:		
	Y-HSP60 + IL-12	Y-HSP60	Control
HKY	6.8	4.8	ND ^d
Y-HSP60	3.1	1.6	1.2
Y-HSP60/CNBr	5.6	5.2	3.1

^a Immunization of mice was performed as described in Materials and Methods. Controls were given injections with PBS.

^b As described in the legend to Table 1.

^c Proliferative responses were determined by [³H]thymidine uptake by 1×10^5 T cells after antigenic stimulation in the presence of 2×10^5 irradiated syngeneic antigen-presenting cells. For details, Table 1, footnote c, and Materials and Methods.

^d ND, not done.

bacterial HSP might directly induce cytokine production in macrophages (50). Hence, HSP may principally contribute to the induction of efficient immune responses.

There is experimental evidence for both of the above-mentioned aspects. First, CD8 T cells reactive with bacterial HSP lyse stressed macrophages which present endogenous HSP at their cell surface (36), and autoimmune diabetes can be elicited by immunization with HSP peptides (17). Second, on the other hand, recent studies have demonstrated that immune responses against microbial HSP mediate protection against infection (49, 54).

In the experimental *Y. enterocolitica* mouse infection model, Y-HSP60 was identified as the dominant antigen for T cells which mediate immunity against yersiniae in adoptive transfer experiments (49). Therefore, we have used Y-HSP60 as a model antigen for vaccination studies. As CD4⁺ $\alpha\beta$ -TCR⁺ gamma interferon-producing T cells are the crucial protective component against *Yersinia* infections (7, 49), a vaccine against *Y. enterocolitica* should induce Th1 responses. Keeping this prerequisite in mind, we selected two approaches for the design of a Y-HSP60 vaccine. First, Y-HSP60-ISCOM were produced. ISCOM, which were first described by Morein et al. (44); are multimeric three-dimensional cage-like structures with a mean diameter of 30 to 40 nm (44). ISCOM have been applied mainly to membrane proteins, because it is difficult to introduce cytoplasmic proteins such as HSP into ISCOM (42). Immunization with ISCOM induces a broad range of immunological responses involving both B and T cells (43). They direct antigens into the major histocompatibility complex class I pathway and thereby induce cytotoxic T-lymphocyte responses (25, 46). Moreover, ISCOM particles induce strong antibody responses, and at least in some models, ISCOM induce mucosal immune responses upon oral or nasal administration (45, 46).

To date, ISCOM have been used only for parasitic and viral antigen preparations, which, after immunization, induced protective immune responses (40). This report is the first to demonstrate that ISCOM can induce immunity against bacterial pathogens, even when applied to cytoplasmic proteins. Thus far, however, it is not clear why nasal or oral administration of Y-HSP60-ISCOM failed to induce systemic immune responses. Whether this is due to an inappropriate immunization protocol (e.g., larger amounts may be required for oral application) or to the immunological events operating in the mucosa-associated lymphoid tissue upon exposure to Y-HSP60 remains to be elucidated. The latter are currently being investigated at our laboratory. On the other hand, we cannot completely exclude that the Y-HSP60 and ISCOM matrix might be in separate particles and that the ISCOM matrix without antigen induced an adjuvant effect, although the small amounts of Y-HSP60-ISCOM required for protective immunity may argue against this possibility.

IL-12 is produced by macrophages and B cells upon stimulation with microorganisms or their products (16, 28, 51). IL-12 promotes growth and cytolytic activity of cytotoxic T lymphocytes and NK cells and stimulates gamma interferon production (20, 21, 35). IL-12 enhances Th1 responses in *Yersinia* infections and, when administered during the acute phase of *Yersinia* infection, renders *Yersinia*-susceptible BALB/c mice resistant to this pathogen (10). Beside its potential therapeutic effect, recent studies suggested that IL-12 can be used as an adjuvant (19, 22). Hence, IL-12 is an essential component of a (subunit) vaccine against *Leishmania major* (56), *Listeria monocytogenes* (60), *Toxoplasma gondii* (22), and *Mycobacterium tuberculosis* (19), all of which are intracellular pathogens. The present study demonstrates that IL-12 can be used as an

adjuvant to promote protective immune responses against enteropathogenic bacteria by using a single protein for immunization. However, we believe that ISCOM might act better than IL-12 as adjuvant in our model.

Even highly *Yersinia*-susceptible BALB/c mice could be protected against a normally lethal *Yersinia* challenge when IL-12 was coadministered with Y-HSP60. Previous studies demonstrated that the susceptibility of BALB/c mice for *Y. enterocolitica* is due to the inability of these mice to generate a rapid and efficient *Yersinia*-specific Th1 response (2, 10). Therefore, we assume that the protective effect induced by Y-HSP60 plus IL-12 is due to the promotion of a Y-HSP60-specific Th1 response in BALB/c mice.

Two further studies which demonstrate that microbial HSP can be used as a protective vaccine without inducing deleterious immune responses have been published. In the first, immunization with *Helicobacter pylori* GroES homolog (small ~10-kDa HSP family) and GroEL-like proteins conferred protection against mucosal infection in mice (18). In this model, IgG1 antibodies are believed to represent the protective component of the HSP-specific immune response because *H. pylori* colonizes the mucosal surface rather than invading the tissues of the host. In an experimental *M. tuberculosis* infection, immunization of mice with J774.G8 cells transfected with HSP65 resulted in presentation of HSP65 fragment by both major histocompatibility complex class I and II molecules (53). Protection induced in the latter model, however, was most efficiently transferred by $\alpha\beta$ TCR⁺ CD4⁻ CD8⁺ cytotoxic T cells (54). This is in contrast to the observations in the *Yersinia* mouse model, in which CD4 Th1 cells represent the most important cell type that confers immunity (49), and reflects the different habitats of the two pathogens. While *M. tuberculosis* is intracellularly located, *Y. enterocolitica* is a predominantly extracellular pathogen (24). *Y. enterocolitica*, however, evades innate host defense mechanisms such as phagocytosis and complement by expression of certain virulence factors such as YadA and Yops (13, 26). Therefore, a Th1-promoted host response is required to overcome *Yersinia* infection.

Taken together, our results demonstrate that vaccination with Y-HSP60 in ISCOM or plus IL-12 induces immunity against *Y. enterocolitica*. However, more detailed studies are now required to fully establish the immunological events involved in these protection mechanisms.

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